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<p>(21) International Application Number: PCT/GB90/00433 (22) International Filing Date: 21 March 1990 (21.03.90)</p> <p>(30) Priority data: 8906776.3 23 March 1989 (23.03.89) GB</p> <p>(71) Applicant (for all designated States except US): AMERSHAM INTERNATIONAL PLC [GB/GB]; Amersham Place, Little Chalfont, Buckinghamshire HP7 9NA (GB).</p> <p>(72) Inventors; and (75) Inventors/Applicants (for US only) : CHARLES, Stephen, Alexander [GB/GB]; 5 Eliot Close, Heydon Hill, Aylesbury, Bucks. HP1 9JB (GB). FINLAN, Martin, Francis [GB/GB]; 7 Ballard Close, Aylesbury, Bucks. HP21 9YU (GB). GARLAND, Peter, Bryan [GB/GB]; Hope Cottage, Sunnyway, Old Bosham, West Sussex PO18 8HQ (GB). POLLARD-KNIGHT, Denise, Vera [GB/GB]; 4 Drury Road, Harrow, Middlesex HA1 4BY (GB).</p>		<p>(74) Agent: PENNANT, Pyers, Stevens, Hewlett & Perkins, 5 Quality Court, Chancery Lane, London WC2A 1HZ (GB).</p> <p>(81) Designated States: AT (European patent), BE (European + patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent), US.</p> <p>Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i></p>									
<p>(54) Title: ASSAY METHOD USING SURFACE PLASMON RESONANCE SPECTROMETRY</p> <table style="margin-left: auto; margin-right: auto;"> <tr> <td style="text-align: center;">Antibody</td> <td style="text-align: center;">T</td> <td style="text-align: center;">Analyte</td> <td style="text-align: center;">●</td> </tr> <tr> <td style="text-align: center;">Protein</td> <td style="text-align: center;"><input type="checkbox"/></td> <td style="text-align: center;">Analyte analogue</td> <td style="text-align: center;"><input type="circle"/></td> </tr> </table> <p>DISPLACEMENT IMMUNOASSAY</p> <p>(a) <u>fixed hapten</u></p> <p>(b) <u>fixed antibody</u></p> <p>(57) Abstract</p> <p>In an assay for an analyte, particularly a hapten, changes in refractive index at a solid surface are monitored by means of surface plasmon resonance spectroscopy. The signal is enhanced by conjugating to an assay reagent a substance capable of giving a strong signal. One example is an enzyme used to catalyse production of an insoluble product. Another example is latex beads. Assay formats include sandwich assay format, and a competition assay format preferably involving displacement of the conjugate.</p>				Antibody	T	Analyte	●	Protein	<input type="checkbox"/>	Analyte analogue	<input type="circle"/>
Antibody	T	Analyte	●								
Protein	<input type="checkbox"/>	Analyte analogue	<input type="circle"/>								

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ASSAY METHOD USING SURFACE
PLASMON RESONANCE SPECTROMETRY

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This invention concerns methods of assaying for analytes using the technique of surface plasmon resonance spectrometry (SPRS). The method is applicable to analytes generally, but is likely to be 10 of particular interest where the analyte is a hapten (a small molecule capable of being bound by antibody but not of itself immunogenic).

The phenomenon on SPR is well known and will not be described in detail. Two effects are known to 15 give rise to SPR, the Woods effect which involves a metallized diffraction grating; and the Kretschmann effect with which this invention is concerned. Reference is directed to EPA 305109 for a discussion of the Kretschmann effect. Briefly, the intensity of 20 monochromatic plane-polarised light (conveniently obtained from a laser) reflected from the interface between an optically transparent material, e.g. glass, and a thin film of metal depends on the refractive index of material in a thin layer, at most a few hundred nm thick, on the downstream side of the metal. Accordingly, by measuring changes in intensity of 25 reflected light an indication can be obtained of changes in refractive index of material on the metal. The intensity of reflected light also varies with the angle of incidence, and reflectivity drops sharply to a 30 minimum at a particular angle characteristic of the equipment. The metal surface is generally of silver, although this is not critical to the invention.

The immunoassay of haptens by Surface Plasmon 35 Resonance Spectrometry (SPRS) poses a particular problem because the haptens are necessarily of low

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molecular weight and therefore cause only very small changes in refractive index when they bind to or dissociate from an antibody-coated SPRS silver-coated surface. This problem is addressed in International
5 Application PCT/GB89/00156.

Two ways around this problem are illustrated in Figure 1 of the attached drawings. In 1 (a), the hapten 10 or analogue is immobilised to the silver surface 12 used for SPRS detection, and binds the
10 corresponding antibody 14. Introduction of free hapten 16 (whose concentration it is wished to determine) displaces antibody by competing with surface bound hapten. This displacement of antibody from the surface is detected as an SPRS signal. In 1 (b), the antibody
15 14 is bound to the surface 12 and binds a conjugate 18 of the hapten 10 or analogue and (typically) a protein 20 of sufficient molecular weight to yield a significant SPRS signal on displacement of the conjugate by added free hapten.

20 However, this arrangement is not always very sensitive. This invention provides a way of increasing the sensitivity.

EPA 276142 describes an SPR assay in which an assay reagent X is chosen to have a high refractive
25 index so as to enhance an SPR signal. In the system described, deposition of the reagent X is monitored by SPR making use of the Woods effect.

The present invention provides a method of assaying for an analyte which is a member of a specific
30 binding pair, by the use of a solid surface carrying immobilised thereon a first reagent which is a member of the specific binding pair, and of a second reagent which is a conjugate of a member of the specific binding pair, at least one of the first and second

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reagents being a specific binding partner of the analyte,

which method comprises incubating a fluid sample containing the analyte with the first and second

5 reagents simultaneously or in any desired order, whereby the conjugate is caused to be present on the solid surface in a manner related to the presence of the analyte in the sample,

characterised in that there is used as the
10 solid surface a metallic layer applied to a block of material transparent to electromagnetic radiation, the presence of the conjugate on the surface is assayed by surface plasmon resonance spectrometry, and the conjugate is selected to be capable of giving a strong
15 SPR signal.

In one embodiment, either (a) the first reagent is an analogue of the analyte and the second reagent is a conjugate of a specific binding partner of the analyte, or (b) the first reagent is a specific
20 binding partner of the analyte and the second reagent is a conjugate of an analogue of the analyte,

and the assay is performed by effecting competition between the analyte and the analyte analogue for binding with the specific binding partner.

25 25 of the analyte, whereby the conjugate is caused to be present on the solid surface in a manner related to the presence of the analyte in the sample.

An analogue of the analyte is a substance which competes with the analyte for binding to a

30 specific binder therefor. Often the analogue will be arranged to be as near as possible or even completely identical to the analyte. The use in assays of analyte analogues is well known.

In a preferred arrangement, the second

35 reagent is reversibly bound to the first reagent as a preliminary step. When the sample is brought into

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contact with the solid surface, competition between the analyte and the analyte analogue causes displacement of a proportion of the second reagent from the detection surface, that proportion being directly related to the
5 concentration of the analyte in the sample. Because that second reagent is a conjugate selected to be capable of giving a strong SPR signal, its displacement from the solid surface is readily and accurately monitored by means of SPRS. An advantage of this
10 arrangement is that the assay is performed simply by bringing the sample into contact with the solid surface, no other reagent being required.

Two embodiments of the invention have been designated (a) and (b). In (a), the first reagent
15 immobilised on the solid surface is an analogue of the analyte, preferably the analyte itself carried on a suitable spacer molecule (generally a macromolecule such as a protein). The second reagent is a conjugate of a specific binding partner of the analyte, most
20 usually of an antibody to the analyte.

In (b) the first reagent, immobilised on the solid surface, is a specific binding partner of the analyte, generally an antibody to the analyte. The second reagent is then a conjugate of the analyte or
25 of an analogue of the analyte.

In one approach, the second reagent is a conjugate with a substance which itself gives a strong SPR signal, as described below.

In another approach, the second reagent is a
30 conjugate with a member of a different specific binding pair which is non-reactive with the analyte. In this case, the assay includes an additional step of bringing into contact with the solid surface a complex of the other member of the different specific binding pair
35 with a substance which itself gives a strong SPR signal. Examples of the different specific pairs

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include the biotin-avidin and biotin-streptavidin systems. Preferably biotin is present in the second reagent.

This additional step is performed during or
5 after the competition step of the assay. The complex may be present in a fluid which is brought into contact with the solid surface. Or the complex may be incorporated in the fluid sample. For example the fluid sample may be caused to flow successively over
10 two solid surfaces, of which the first carries the complex in soluble form and the second is the SPR detection surface.

The substance may be one having a high refractive index. This may be a molecule or particle
15 with a high refractive index or a large size, to confer a higher refractive index on the reagent as a whole thus giving rise to a larger SPR signal than would be the case with an unmodified molecule. Possible substances include heavy substances (e.g. metal ions or
20 higher halogens) highly electronically delocalised species (e.g. polycyclic aromatics or dyes), metal or metal oxide particles such as titania particles, or high refractive index organic species such as ferritin.

Alternatively, the substance may be one
25 having a low refractive index, i.e. a refractive index lower than that of the environment close to the solid surface.

Alternatively, the substance may be an enzyme
30 which is caused to catalyse a reaction resulting in the production of a reaction product which is deposited on the solid surface. It is necessary that the reaction product resulting from the action of the enzyme be immobilised on or exceedingly close (i.e. within a thin layer of a few hundred nanometers or less) to the metal
35 surface. The reaction product may have a refractive index which is higher or lower than the material

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present in that thin layer. Examples of suitable enzymes are:-

- a) Peroxidase, with H₂O₂ and diamino-benzidine (DAB) as substrates. The latter is converted to an insoluble product.
- b) Certain oxidoreductases with NAD(P)H and a tetrazolium salt as substrate. The latter is converted to an insoluble product (a formazan). Other reductant and dye combinations exist. (See F P Altmann, 1972, "An introduction to the use of tetrazolium salts in quantitative enzyme cytochemistry" published by Koch-Light Laboratories, Colnbrook, Bucks, England).
- c) Certain catalase enzymes are known to generate gas, bubbles of which can be retained close to the solid surface. When the second reagent is a conjugate of a member of the specific binding pair with an enzyme, the assay may conveniently be performed in a sandwich assay format. In this case, both the first and second reagents are specific binding partners of the analyte (i.e. antibodies when the analyte is an antigen or hapten). This is in contrast to the displacement assays described above which are necessarily performed in a competition assay format.

The assay performed by the method may be qualitative, i.e. simply to detect the presence or absence of an analyte in a sample, or quantitative. For quantitative assays, measurements may be made of the rate of change of reflectivity, and/or of the absolute reflectivity at a given time. Contact between the fluid medium and the solid surface may be static, but is more preferably dynamic e.g. by the fluid medium being caused to flow across the metal surface.

The nature of the analyte is not critical.

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However, for the reasons given above, the invention is of particular value when the analyte is a hapten. The first reagent may be immobilised on the surface of the metal e.g. silver layer by conventional means, e.g.

5 covalent bonding or simply physical adsorption. A biotinylated reagent may be immobilised on a metal surface to which streptavidin has been bound. Indeed immobilisation may involve any binding pair, including DNA/DNA or antibody/antigen.

10 Reference is directed to Figures II, III and IV of the accompanying drawings which illustrate aspects of the invention, and in which:

Figure II comprises two reaction diagrams illustrating one embodiment;

15 Figure III is a reaction diagram illustrating another embodiment, and

Figure IV shows the final state of the solid surface when an enzyme is used.

20 Figure II (a) shows the preliminary step in an assay for an analyte in a sample. At the outset, an analogue 22 of the analyte is irreversibly attached to a silver layer 28, and an antibody conjugate 24 is reversibly bound to the analogue.

Figure II (b) shows a similar arrangement.

25 At the outset, antibody 26 to the analyte is irreversibly attached to the silver layer 28 and an analyte analogue conjugate 30 is reversibly bound to the antibody.

Both systems operate in the same way. On 30 addition of a sample 32 containing the analyte 34, a proportion of the conjugate molecules is displaced from the silver layer, that proportion being directly related to the concentration of the analyte in the sample.

35 Figure III shows a two-step assay. At the start, antibody 36 to the analyte is irreversibly

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attached to a silver layer 38, and a conjugate of an analyte analogue 40 with biotin 42 is reversibly bound to the antibody. On addition of a sample 44 containing an analyte 46, a proportion of the conjugate molecules is displaced from the silver layer, that proportion being directly related to the concentration of the analyte in the sample.

A fluid 48 containing a complex of streptavidin 50 with HRP enzyme 52 is then brought into contact with the silver layer. The streptavidin binds to the biotin to an extent inversely related to the concentration of the analyte in the sample.

The final step of the assay is shown in Figure IV. When diamino-benzidine (DAB) and hydrogen peroxide (H_2O_2) are brought into contact with the silver layer, the HRP enzyme catalyses reaction between them leading to formation of an insoluble product which accumulates adjacent the silver layer 38 and gives rise to a strong SPR signal which is easily monitored.

In place of the HRP enzyme 52, there could have been used a high refractive index substance, such as ferritin or a titania particle, as described above.

In another embodiment, an enzyme may be irreversibly bound on the solid surface in an inactive form. When the fluid sample is brought into contact with the solid surface, analyte in the sample may activate the enzyme which may then be used to generate an SPR signal in the manner shown in Figure 4. The enzyme thus activated by the analyte may itself be one which generates an insoluble product; or may be one which generates a substrate for such an enzyme.

The following Examples illustrate the invention

In each of Examples 1 and 2, the analyte is human immunoglobulin, and a sandwich assay format is used. The first reagent, immobilised on a silver

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surface, is an anti-human immunoglobulin antibody. The second reagent is an (anti-human immunoglobulin antibody) - enzyme conjugate. In a first step, a sample containing the analyte is brought into contact
5 with the silver surface, whereby the analyte binds to the immobilised first reagent (present in excess).

In a second step, a solution of the second reagent is brought into contact with the silver surface, whereby the second reagent becomes bound on
10 the solid surface to an extent proportional to the amount of analyte in the sample. This gives rise to a small but measurable SPRS signal. To amplify the signal, a substrate for the enzyme is supplied to cause an insoluble reaction product to be deposited on the
15 silver surface.

In Examples 1 and 2, the first step is assumed to have taken place, and the second step is performed by applying the second reagent direct to the silver surface. The second step, demonstrated in the
20 Examples, is the key to the method.

Example 1

An anti-human immunoglobulin- β galactosidase
25 conjugate was diluted to 36nM in 10mM sodium phosphate pH 7.4 and 1ml pumped across a silver slide at 8 μ l/sec. The shift in the angle at which SPR occurs (change in reflectivity) was monitored on binding the conjugate. 3mls of 10mM sodium phosphate pH7.4 was then manually
30 injected across the slide to wash the surface.

A block of sheep immunoglobulin (2 μ M, 1ml) in 10mM sodium phosphate pH7.4 3mM MgCl₂, was injected across the slide, followed by a wash of 3mls of 10mM sodium phosphate pH7.4, 3mM MgCl₂, 0.5% BSA. The
35 substrate solution (VLM from PPR Diagnostics, Kings College, London) diluted to 0.4mM in 10mM phosphate

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buffer pH7.4 was then flowed at 3 μ l/sec across the slide and the change in reflectivity monitored with time. A wash step of 3mls of 10mM sodium phosphate was then performed.

5 The results from two experiments are shown in the table below. There is at least a five fold enhancement of the signal on addition of the substrate.

Example 2

10

An anti-human immunoglobulin-alkaline phosphatase conjugate diluted to 0.125nM and 60pM in 10mM Tris/HCl pH 9.5, 3mM MgCl₂ (dilution buffer) was flowed across a silver coated slide at 4 μ l/sec and the 15 change in reflectivity monitored. Following a wash step with 3mls of this same buffer, the slide was blocked with 2 μ M sheep immunoglobulin in 10mM Tris/HCl pH 9.5, 3mM MgCl₂ (1ml manual injection). A further wash step (3mls of dilution buffer) was performed and 20 the enzyme substrate solution (3.3mg/ml nitro-blue tetrazolium, 1.65mg/ml 5-bromo-4-chloro-3-indoyl-phosphate in dilution buffer) then flowed across the slide at 3 μ l/sec. The change in reflectivity with time was monitored.

25 The table below shows the results for 0.125nM and 60pM anti-human immunoglobulin-alkaline phosphatase conjugate respectively. The addition of the enzyme substrate significantly enhances the sensitivity of the assay.

30

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Final Change in Reflectivity (%)

	Bind of enzyme conjugate	Addition of enzyme substrate
5		
10	Example 1 36nM β -glactosidase- anti-human immunoglobulin	7 37
15	Example 2 0.125nM anti-human immunoglobulin -alkaline phosphatase	4 51
20	60 pM anti-human immunoglobulin -alkaline phosphate	2.2 28

Example 3

25 A Displacement Assay for Human Chorionic Gonadotrophin (HCG) using a Refractive Index Probe

10mM sodium phosphate buffer, pH 7.4 (buffer 1) was pumped across the silver slide and the change in SPR angle monitored. A solution of 166nM human immunoglobulin-HCG conjugate diluted in buffer 1 was then bound to the silver surface by pumping across the surface at 8 μ l/sec. Following a wash step with buffer 1, the silver surface was then blocked by manual injection of 2 μ M sheep immunoglobulin diluted in 10mM sodium phosphate buffer, pH7.4 containing 0.5% bovine serum albumin (buffer 2) across the silver slide. A

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mouse anti-HCG monoclonal antibody, free in solution or linked onto 55nM polystyrene refractive index probes (enhanced sensitivity), was then bound to the previously immobilised HCG-conjugate by pumping a 100nM 5 solution of the anti-HCG antibody diluted in buffer 2 across the silver slide. The silver slide was then washed with buffer 2. 1 μ M HCG diluted in buffer 2 was then added and displacement of the anti-HCG antibody from the silver surface was observed by SPR. As shown 10 in the table below both the rate of displacement and the final change in the shift in the SPR angle were greater when the anti-HCG antibody was attached to the 55nM polystyrene refractive index probes.

15

DISPLACED MOLECULE

RATE* -0.0322 -0.0043

25

FINAL CHANGE** -7.4% -1.2%

30

* change in reflectivity per second
** change in reflectivity at the end of the assay

35

CLAIMS

5

1. A method of assaying for an analyte which is a member of a specific binding pair, by the use of a solid surface carrying immobilised thereon a first reagent which is a member of the specific binding pair,
10 and of a second reagent which is a conjugate of a member of the specific binding pair, at least one of the first and second reagents being a specific binding partner of the analyte,

15 which method comprises incubating a fluid sample containing the analyte with the first and second reagents simultaneously or in any desired order, whereby the conjugate is caused to be present on the solid surface in a manner related to the presence of the analyte in the sample,

20 characterised in that there is used as the solid surface a metallic layer applied to a block of material transparent to electromagnetic radiation, the presence of the conjugate on the surface is assayed by surface plasmon resonance spectrometry, and the
25 conjugate is selected to be capable of giving a strong SPR signal.

2. A method as claimed in claim 1, wherein either a) the first reagent is an analogue of the analyte and the second reagent is a conjugate of a specific binding partner of the analyte, or b) the first reagent is a specific binding partner of the analyte and the second reagent is a conjugate of an analogue of the analyte, and the incubation step is performed so as to effect competition between the
30 analyte and the analyte analogue for binding with the specific binding partner of the analyte.

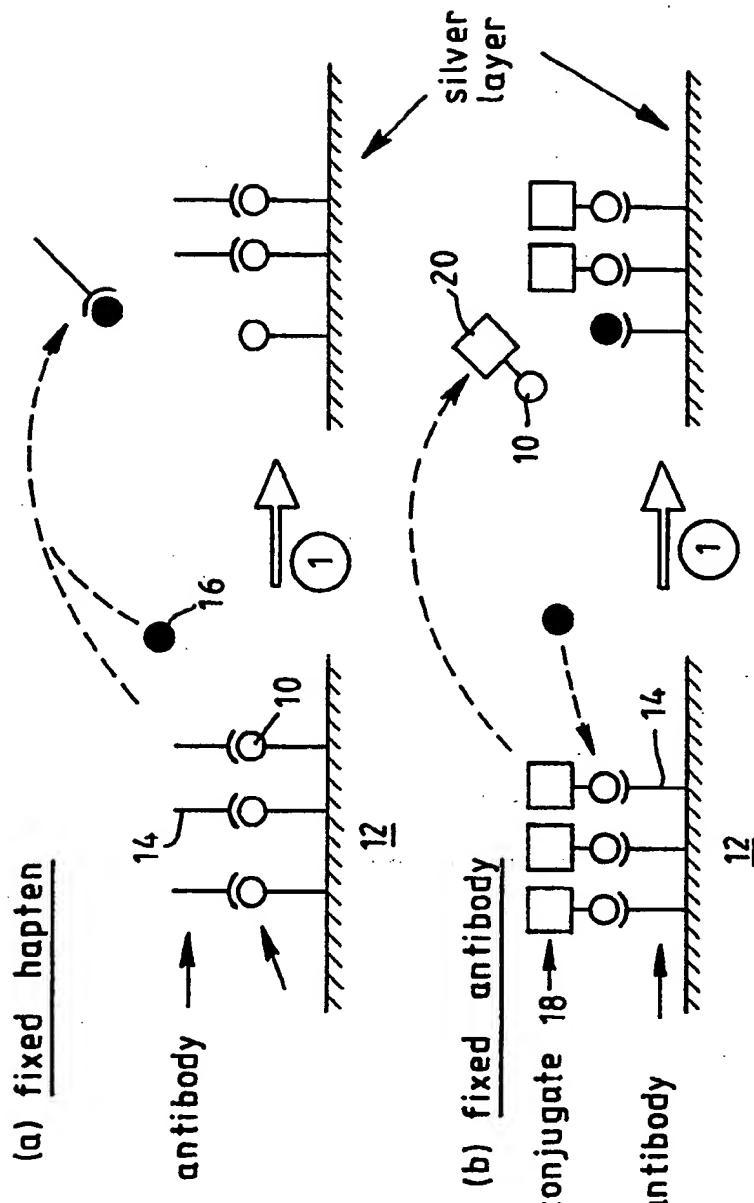
- 14 -

3. A method as claimed in claim 1 or claim 2, wherein the conjugate is with a substance which gives a strong SPR signal.
4. A method as claimed in claim 1 or claim 2, 5 wherein the conjugate is with a member of a different specific binding pair which is non-reactive with the analyte, and the assay includes the additional step of bringing into contact with the solid surface a complex of the other member of the different specific binding 10 pair with the substance which gives a strong SPR signal.
5. A method as claimed in claim 3 or claim 4, wherein the substance is one having a high refractive index.
- 15 6. A method as claimed in claim 1 or claim 2, wherein the second reagent is a conjugate of a member of the specific binding pair with an enzyme, which enzyme is caused to catalyse a reaction resulting in the production of a reaction product which is deposited 20 on the solid surface.
7. A method as claimed in any one of claims 1 to 6, wherein the second reagent is reversibly bound to the first reagent and the sample is then brought into contact with the solid surface, whereby a proportion of 25 the second reagent, directly related to the concentration of the analyte in the sample, is displaced from the detection surface.
8. A method as claimed in claim 6, wherein a sandwich assay format is used.
- 30 9. A method as claimed in any one of claims 1 to 8, wherein the analyte is a hapten.

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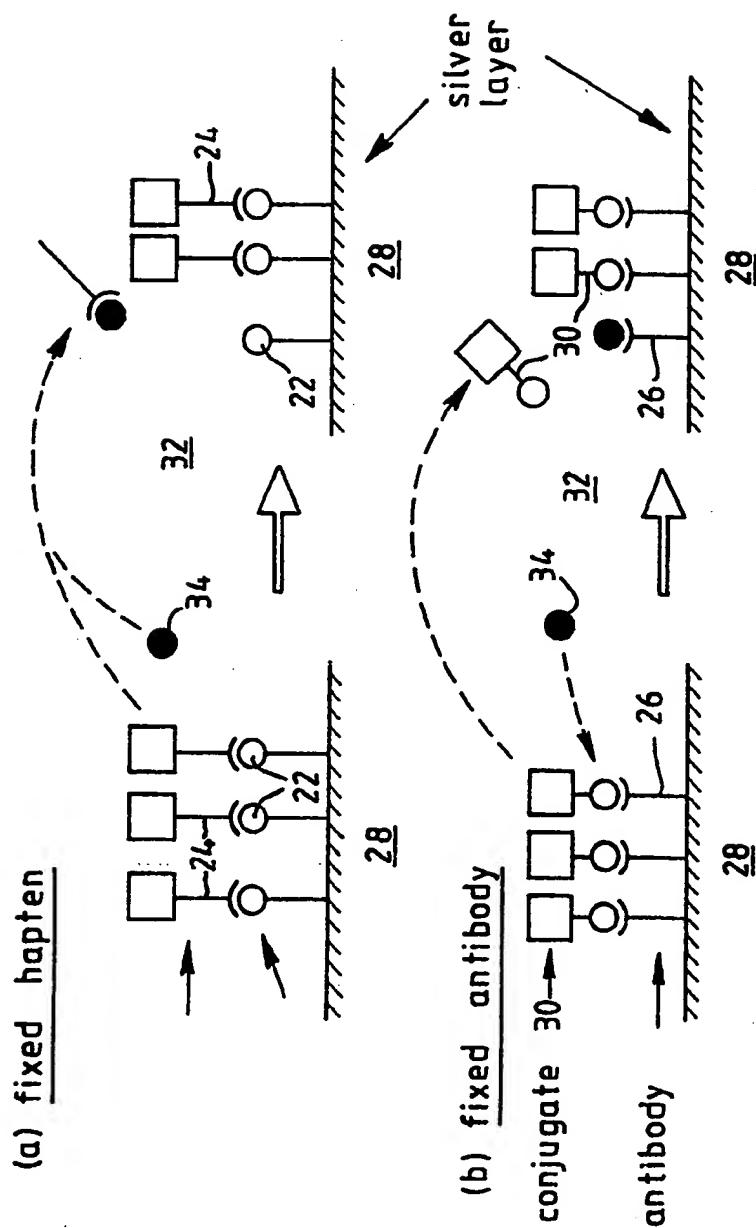
Antibody  Analyte ●
 Protein  Analyte analogue ○

FIG. 1 DISPLACEMENT IMMUNOASSAY



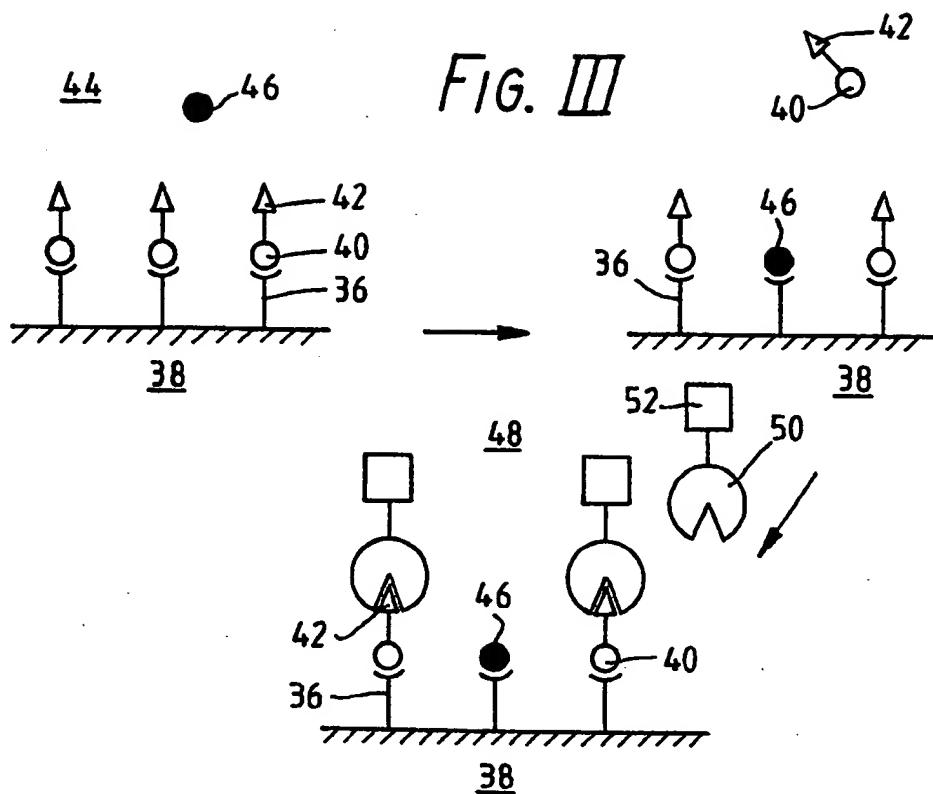
Antibody Analyte ●
 Conjugate or Analyte analogue ○
 (second reagent)

FIG. II DISPLACEMENT IMMUNOASSAY



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FIG. III



Antibody

1

Analyte

1

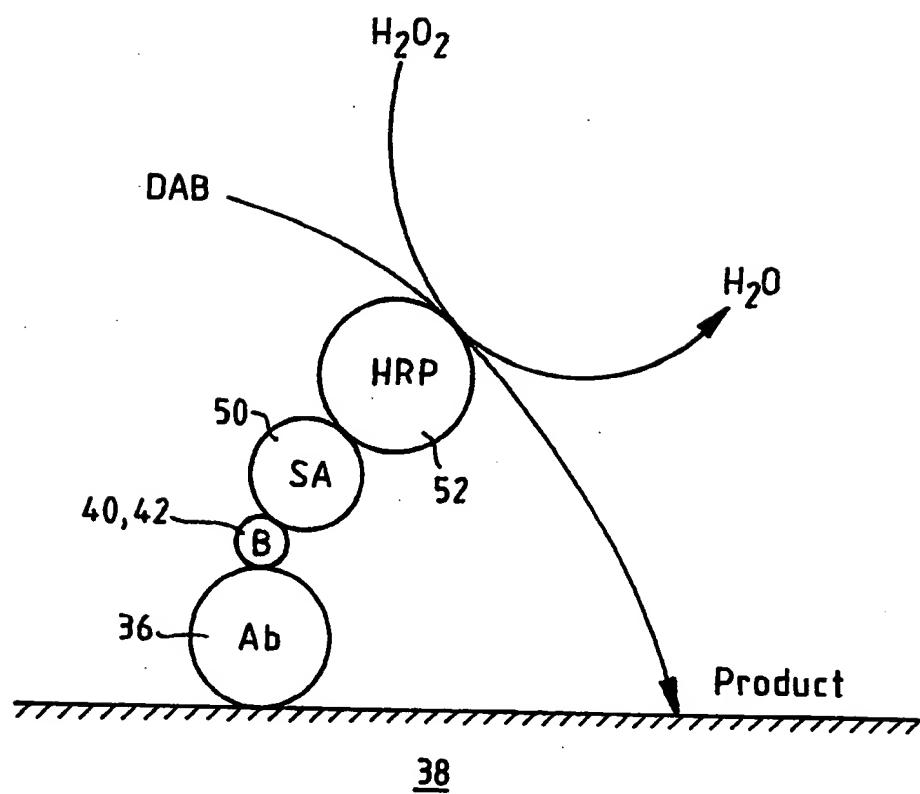
Conjugate of biotin and analyte analogue

1

Conjugate of enzyme
and streptavidin

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FIG. IV

38

INTERNATIONAL SEARCH REPORT

International Application No. PCT/GB 90/00433

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁶

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC⁵: G 01 N 33/543, G 01 N 21/75

II. FIELDS SEARCHED

Classification System :	Minimum Documentation Searched ⁷	
	Classification Symbols	
IPC⁵	G 01 N 33/553, 33/543, 21/75, 21/55	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁸		

III. DOCUMENTS CONSIDERED TO BE RELEVANT*

Category ⁹	Citation of Document, ¹⁰ with indication, where appropriate, of the relevant passages ¹¹	Relevant to Claim No. ¹²
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A EP, A, 0276142 (ARES SERONO RESEARCH) 1
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 1 March 1989
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 pages 920(A)-931(A)
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./. .

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- "A" document defining the general state of art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
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- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- "Z" document member of the same patent family

IV. CERTIFICATE

Date of the Actual Completion of the International Search

4th June 1990

Date of Mailing of this International Search Report

27 JUL 1990

International Searching Authority

EUROPEAN PATENT OFFICE

Signature of Authorized Officer

Mme N. KUIPER

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, ** with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	EP, A, 0028132 (DYNASCiences CORP.) 6 May 1981 see page 3, first paragraph; page 5, down to "dinitrobenzenes" -----	1

**ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO.**

GB 9000433
SA 35707

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EPO file on 24/07/90
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Patent document cited in search report	Publication date	Patent family member(s)		Publication date
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